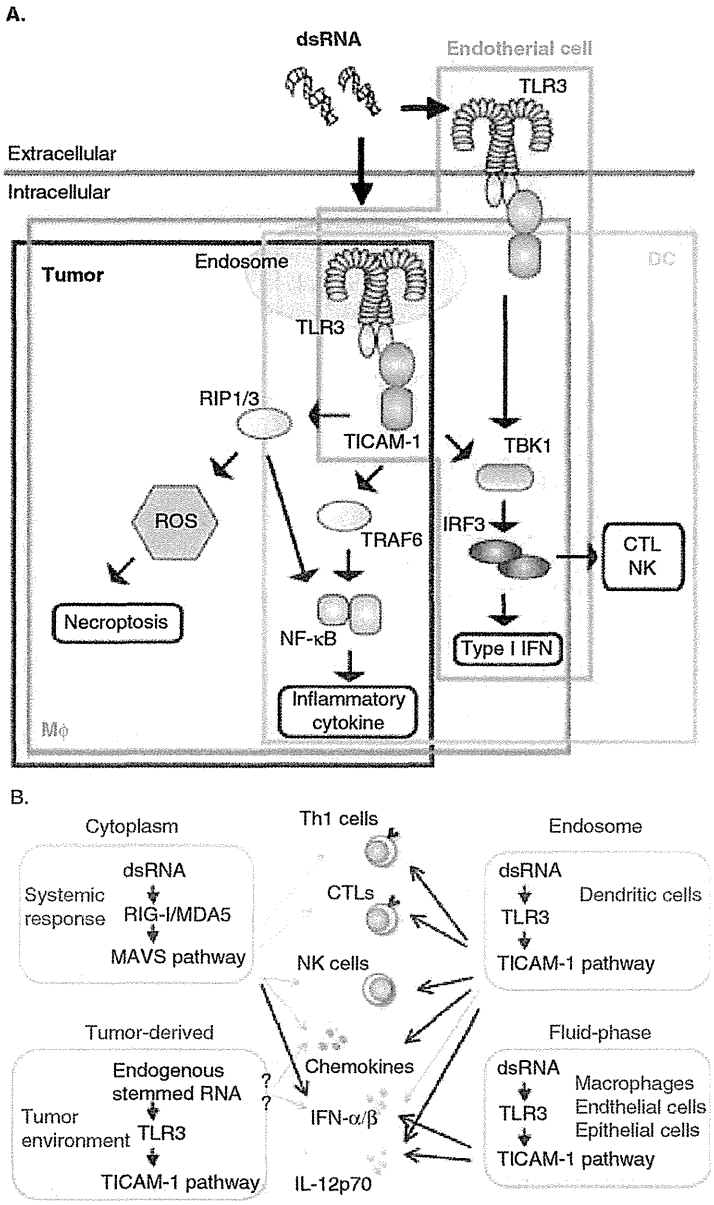

Figure 1. A. Cell type-specific activation of the TLR3/TICAM-1 pathway. Endothelial cells, epithelial cells and some macrophage subsets of human and mouse express TLR3 on the surface of the membrane and extracellularly sample naked double-stranded or stem/bulged RNA (red box). Tumor cells usually express TLR3 in the endosome and some cells activate the RIP1/3 pathway in response to dsRNA (black box). Most myeloid cells express TLR3 in the early endosome and take up debris-encapsulated dsRNA (blue and brown boxes). Cell death is induced in some tumor cells through the RIP1/3 pathway, which causes liberation of RNA-containing debris. Macrophages have unique properties of RIP1/3 and release DAMP. See the text for the functional properties of dendritic cell TLR3. **B.** A variety of output induced by dsRNA. Viruses produce dsRNA in the cytoplasm of infected cells during replication, and the cytoplasmic dsRNA is sensed by RNA sensors, RIG-I/MDA5 (left top panel). The cytoplasmic sensors contribute to production of robust type I IFN, leading to systemic cytokinemia, while they only weakly trigger other effectors without participation of the IFNAR pathway. On the other hand, dsRNA, either naked or encapsulated, can be incorporated into the endosomes of dendritic cells to induce cellular and soluble effectors (right top). Roles of surface-expressed TLR3 and endogenous stemmed RNA in this context still remain poorly characterized (bottom panels).

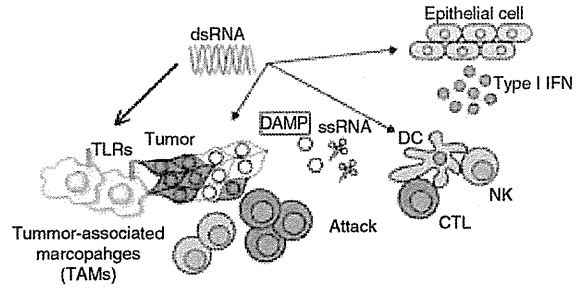

Figure 2. dsRNA-mediated inflammation modulates tumor microenvironment. A dsRNA or stemmed ssRNA affects tumor environment by acting on RNA sensors in epithelial cells, dendritic cells, tumor-infiltrating macrophages and tumor cells. Immune cells infiltrating the tumor mass may cause necroptosis of tumor cells. Tumor cells undergoing necrosis liberate DAMPs and debris containing Ag and nucleic acids with modified structural signatures of stem and bulge. These signatures can activate endosomal TLR3 in dendritic cells to promote the inflammatory response. Tumors in some cases benefit from the inflammatory response and in other cases regress in response to inflammation, and the mechanism determining this switch remains to be clarified.

which in turn recruits kinases TBK1 and IKK to the N-terminal domain of TICAM-1 [25]. The TICAM-1 complex thereafter dissociates from TLR3 and forms cytoplasmic speckles which are distinct from endosomes [26]. The kinase substrates IRF-3 and NF-κB are activated within the speckles, suggesting that the latter contain kinases and their substrates along with TICAM-1 [26,27]. After phosphorylation, IRF-3 migrates from the speckled region to the nucleus and acts as a transcription factor to induce type I IFN [27]. Without activation of the MAVS pathway, production of type I IFN is therefore a primary endpoint of dsRNA-mediated TLR3 activation in most cell types.

Notably, in some tumor cells and macrophages, ligand-stimulated TLR3 can facilitate RIP1 activation via the C-terminal TICAM-1 pathway, resulting in apoptosis or necroptosis (Figure 2) [28,29]. Furthermore, the TICAM-1 pathway triggers a chemokine/cytokine cascade via NF-κB activation that facilitates tumor progression in concert with the tumor microenvironment [30,31]. On the other hand, antigen-presenting cells (DC and Mf) express high amounts of TLR3, together with MHC and co-stimulatory molecules, and interact positively or negatively with lymphocytes. The primary function of myeloid cell TLR3 is to drive activation of NK cells by up-regulating surface-expressed NK-activating ligands (cell cell contact-mediated activation) [32] or induction of IL-12 and IL-15 (cytokine-mediated activation) [33]. Another function of myeloid cell TLR3 is to induce cross-presentation in DC and cross-prime antitumor CD8 T lymphocytes (CTL) [34]. Together, these functions of TLR3 are crucial for induction of antitumor cellular immunity (Figure 2).

4. Therapeutic TLR3 agonists

Three synthetic dsRNAs which harbor therapeutic potential as TLR3 agonists have been developed from polyriboinosinic polyribocytidylic acid (polyI:C), originally synthesized in the late 1960s to mimic viral responses [35]. IPH-3102, a dsRNA of unknown structure, appears to have a similar function to polyI:C [36]. In addition to these classical dsRNAs, single-stranded RNAs with nuclease-resistant stems are potential TLR3 agonists (Table 2) [37,38].

Ampligen (also known as rintatolimod) is a synthetic dsRNA consisting of polyI:C with one mismatch every 12 C, designating poly(I:C12U). It acts on DCs to induce tumoricidal effects, resulting in tumor growth retardation *in vivo* [39]. Ampligen, though has not been immunobiologically well-characterized as a specific agonist for TLR3, appears to operate in a multimodal fashion, encompassing the activation of natural killer (NK) cells, the proliferation of CTL, as well as direct cytostatic/cytotoxic effects on cancer cells. Hence, Ampligen targets a putative dsRNA sensor, most likely TLR3 across various cell types. Ampligen may not be a ligand for RIG-I/MDA5 [39].

Hiltonol (polyI:CLC) is a particular formulation of polyI:C that contains carboxymethylcellulose and poly-L-lysine as stabilizing agents [40]. Hiltonol is less vulnerable to degradation by serum nucleases or high temperature than Ampligen [41]. Hiltonol significantly elevates the levels of circulating IFNs in monkeys under conditions where an equivalent dose of Ampligen did not [40]. This has been attributed to the ability of Hiltonol to act as a ligand for both TLR3 and MDA5 [7,8] to exert potent immunostimulatory effects. Robust increases of serum type I IFN have been observed in multiple preclinical models, including mice and monkeys, although these are accompanied by an increased risk of side-effects [40-44]. Phase I/II clinical trials have been designed with patients having multifarious malignancies to assess the safety and efficacy of Hiltonol [45]. In general, these trials have concluded that low doses of Hiltonol are not particularly toxic in terms of cytokine induction and are moderately efficient in boosting antitumor immune responses. To date, ~ 20 Phase I/II clinical trials have suggested that Hiltonol is adaptable to immunotherapy for cancer in most cases, including brain tumor, malignant melanoma, breast cancer, and colorectal cancer [45].

Polyadenylic polyuridylic acid (polyA:U) is a synthetic dsRNA with immune-enhancing function *in vivo* on 1967 [46]. polyA:U was later found to stimulate TLR3 in Flt3-derived conventional DCs to generate IL-12 [47]. RIG-I/MDA5 recognizes only high amounts of polyA:U [46,48]. When combined with anticancer vaccines, poly(A:U) promotes Th1 responses that control tumor growth and are associated with the establishment of immunological memory [49]. On the other hand, poly(A:U) has protumor functions [50], because it stimulates TLR3 expressed on tumor cells to induce tumor cell proliferation [28,51-53]. There are currently no

Table 1. TLR repertoire in human dendritic cells.

Human TLRs	Monocyte	Freshly isolated mDC (BDCA1+)	Freshly isolated mDC (BDCA3+)	*In vitro*-differentiated cells pDC (BDCA4+)	*In vitro*-differentiated cells Monocyte-derived DCs
mAb					
TLR1 (1.136)	++	+	++	-	+
TLR2 (2.45)	++	++	+	-	++
TLR3 (3.7)	-	++	+++	-	++
TLR4	++	+	-	-	+
TLR6 (6.127)	++	+	++	-	+
TLR7		-	-	+	-
TLR8	+	+	+/-	-	+
TLR9	-	-	-	+	-

+: Protein or mRNA expression; Nucleotide-recognizing TLRs, TLR3, 7, 8 and 9 reside in intracellular compartments. MAVS pathway is ubiquitous while TICAM-1 pathway limitedly works in myeloid, epithelial and endothelial cells.

Table 2. Host response to RNAs and other DAMPs.

PAMP/DAMP receptors	
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5'-PPP-RNA	RIG-I
Endosomal > 140 bp dsRNA	TLR3
Fluid-phase dsRNA	TLR3
Encapsulated virus RNA	TLR3
Bulged or stemmed RNA	TLR3
Self nucleic acids (DAMP?)	
Modified self mRNA	TLR3
Some miRNA	TLR3
Denatured ssRNA	TLR3
Self molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14,TLR2/4
HSPs	CD14,TLR2/4
S100 proteins	RAGE

clinical trials evaluating its efficacy for either oncological or cancer-unrelated indications.

More exact information about clinical trials and oncological indications of these TLR3 agonists has been published by Galluzzi *et al.* [45].

5. Specific ligands for TLR3 without activation of the MAVS pathway

dsRNA and its synthetic analog, polyI:C, have long been known to be potent type I inducers [54] and modulators for cellular immunity [55]. Indeed, mouse and human versions of TLR3 recognize dsRNA and transduce TICAM-1 signals for NF-κB and IRF-3 activation. Type I IFN/cytokine and cellular immunity induced by the cytoplasmic dsRNA receptors RIG-I/MDA5 and NLRP3 have been identified more recently [45,48,56]. The type I IFN production induced by dsRNA is largely attributable to the MAVS pathway rather than the TICAM-1 pathway [7]. Furthermore, KO mice studies with *in vivo* administration of polyI:C have suggested that dsRNA contributes to NK cell activation and CTL proliferation even in MAVS-/- and IFNAR-/- mice, the initial response to which is independent of MAVS- or IFNAR-mediated type I IFN production [34,57]: i.e., tumor-specific NK cell and CTL can be induced without increasing serum type I IFN level in mice. Hence, the activation of cellular immunity occurs irrespective of the serum level of IFN in tumor-bearing host, although IRF-3 is essential for cellular immune activation [57].

Regarding the question as to through which target receptor, MDA5 or TLR3, polyI:C induces antitumor cellular immunity, evidence suggests that it is TLR3 [32,34]. Previous studies demonstrated that both MDA5 and TLR3 were equally associated with initiation of cellular immunity in response to i.p. injection of polyI:C + Alum in mice [58]. However, initial IRF-3 activation in myeloid cells is closely linked to NK cell activation but not robust IFN induction [57-59]. In syngenic mouse tumor-implant models, TLR3-TICAM-1 is more important than MDA5-MAVS for CD8α$^+$ DC to evoke antitumor cellular immunity below the protumorigenic polyI:C dose [32,34].

The optimal *in vivo* doses for induction by polyI:C of type I IFN, NK activation or CTL induction (cross-priming) are as yet unknown. It is likely that the optimal dose for inducing type I IFN, largely attributable to the RIG-I/MDA5 pathway, differs from those for facilitating NK activation and/or cross-priming induced by human DC [19], particularly a subset of a human counterpart of mouse CD8α$^+$ DC, namely CD141$^+$ DC. In both human and mouse DCs, 10 μg of polyI:C activates NK cells to kill tumor cells *in vitro* [60]. However, the dose discrepancy appears to cause different immune responses between human and mouse with respect to *in vivo* polyI:C administration. In the C57BL/6 mouse, 1 μg of i.p. injection of polyI:C per mouse is sufficient to induce type I IFN and IFN-inducible genes in spleen cells, but is insufficient

for causative NK/CTL activation to effect regression of implant tumors [32,34,61]. Likewise, in human volunteers, 1.6 mg of s.c. polyI:C has been shown to induce type I IFN in whole blood [62]. It is currently unknown whether this dose is sufficient for activation of NK/CTL in humans. The reported doses were restricted by the toxicity of polyI:CLC, and may have been sufficient for RIG-I/MDA5 activation followed by the feedback activation of the amplifiable IFNAR pathway in mice and humans, however, the dose has not been determined to optimize IRF-3-dependent NK/CTL activation by Ag-presenting DC.

NK cell activation requires > 10 μg of polyI:C per mouse, although the quality of polyI:C, including the average length of the duplex region, varies and critically affects the optimal dose for induction of cellular immunity [62,63]. For induction of cross-priming in mice, > 50 μg/20 g is actually required by i.v. or i.p injection. With regard to s.c. injection of dsRNA, several shots in different areas would be ideal for administration of the dsRNA reagent. If high-dose administration of dsRNA is also mandatory for induction of cellular immunity in humans, the dose 1.2 1.6 mg/volunteer would be a short dose in humans. If the dose limitation of polyI:C in human trials is mostly due to side-effects such as cytokinemia and protumor activity, the development of less toxic RNA reagents is indispensable for facilitating human immunotherapy.

There are several points of concern in the context of high-dose polyI:C therapy. Firstly, the likelihood of a cytokine storm is increased in healthy volunteers receiving > 1.6 mg polyI:C due to systemic activation of the MAVS pathway. Erythema, arthralgia and general malaise have been reported and may be secondary to elevated type I IFN [64]. The other point concerns the protumor activity of the TICAM-1-RIP1 pathway. Appropriate doses that neither activate the RIP1 pathway in tumor cells nor induce tumor growth should be chosen for antitumor therapy. Moreover, the duration of the effects is currently also unknown, although a single-shot dsRNA has only a short duration over ED_{50}. IFN-α/β levels may be kept high, being sustained by the IFNAR pathway [65]. TLR3 is endosomally expressed in myeloid DCs as well as in tumor cells. The types of cell involved in the immune response against high-dose polyI:C remain undetermined in humans, and the role of RNA-sensing receptors in other cell types therefore warrants further exploration.

6. Other RNA derivatives in tumor environment

Recent reports have suggested that single-stranded (ss)RNAs with incomplete stems serve as ligands for TLR3 [37,38]. ssRNA with a ~ 200 bp duplex may act as a TLR3 agonist without activation of MDA5 [24,63]. As mentioned above, the capacity of ssRNA to activate TICAM-1, but not MAVS, makes it suitable for antitumor immunotherapy, since it has only marginal cell-proliferative activity but fully activates NK cells and CTL in relevant tissues with induction of only low levels of IFN. The results are promising in the context of the synthesis of TLR3-specific ligands which do not participate in the MAVS pathway, and which can be applicable to humans without marked toxicity.

It has been believed that viral dsRNA is liberated from virus-infected cells through cell death events, apoptosis or necrosis. Oncogenic viruses may trigger death signals by activating cytoplasmic RNA sensors in transformed cells. An EB virus RNA with an incomplete stem, named EBER, also activates TLR3 [66] and, together with RIG-I, induces live signals and sometimes accelerates tumorigenesis in infected hosts [67]. Alternatively, transformed cells release live signals in the form of type I IFN and proinflammatory cytokines (IL-6, IL-12, TNF-α, etc.), which are liberated through IRF-3/7 and NF-κB activation as the output from living virus-infected cells. TLR, NLR and other cytosolic nucleic acids sensors are closely associated with RNA recognition (**Table 2**), and inflammation states are therefore fundamentally variable and individually modified by these factors [56,68]. It is notable that type III IFN (IFN-λ) is also generated via the TICAM-1 pathway in $CD8\alpha^+$ DC in mice and human $CD141^+$ DC in response to polyI:C [69]. Yet, in other cell types such as hepatocytes, the MAVS pathway participates in IFN-λ production [70].

In addition, tumor cells may liberate self mRNA, miRNA and other endogenous noncoding RNAs (**Table 2**), which become TLR3 ligands through conformational alterations which result in the formation of incomplete stems [71,72]. These self RNAs allow TLR3-positive host cells to induce IFNs and chemokines (**Figure 1B**). Once type I IFN and IFN-γ are robustly produced, the synergistic function of these IFNs results in the induction of IFN-stimulated genes (ISG) in the tumor and surrounding cells, including CXCL10 (IP-10) and CCL5 (RANTES) [72,73]. CXCR3 ligands (CXCL9, 10 and 11) are also expressed by these cells [73]. Since CXCR3 is mainly expressed on activated T and NK cells, these cytotoxic effectors converge upon the inflammatory nest, which includes the tumor microenvironment as well as secondary-affected organs. The tumor microenvironment is likely to be modified by these mediators in conjunction with cellular immune response.

These immunological aberrations may coincide with ecological environmental factors besides viral infection. Indeed, in mouse models, UV-B irradiation effects conformational changes in dermal mRNA to convert nonstimulatory mRNA to active TLR3 ligands by forming with incomplete stems [37], which then activate the TLR3 pathway, similar to virus-derived RNA [38], resulting in inflammatory sunburn. In any case, RNAs with bulged stems are functional as TLR3 agonists to induce IFN-α/β and possibly cellular immunity [37,38,66].

Whether endogenous TLR3 ligands are tumorigenic or tumoricidal remains to be determined. Necrosis-like cell death occurs in a cell type-specific manner as a result of death

signaling and liberates damage-associated molecular patterns (DAMP) of autologous TLR3 ligand (Table 2). Levels of RNA-derived TNF-α and its receptor, TNFR1, have been implicated in this process [74]. The RIP1/RIP3 complex, termed the necrosome, is responsible for switching between apoptosis and necroptosis [75,76]. TICAM-1 and RIP1 may be involved in the virus-derived as well as tumor cytolysis [77], although the possible involvement of RIG-I/MDA5 in cell death cannot be ruled out in some cases of viral infection [78]. DAMP and stemmed RNA can be liberated from tumor-infiltrating Mf as well as necroptotic tumor cells [77]. TNF-α and IL-6 are the pro-inflammatory cytokines released from Mf. A reported feature of exogenous dsRNA in the context of the tumor environment is to damage tumor cells by activation of Mf or the TLR3 pathway in these cells [10]. However, in tumor microenvironment containing tumor-infiltrating Mf, the role of the endogenous stemmed RNA in tumor progression and immune cell activation is the next issue to be elucidated (Figure 2).

7. Cellular immunity induced in tumor microenvironment

Once DC or Mf responds to an unusual innate dsRNA signature, cellular immunity is provoked against tumor cells with irregular modification of RNA-sensing pathways by these immune enhancers (Figure 2). NK cells and CTL are known to be associated with maturation of myeloid DCs after stimulation with dsRNA [79-81]. DCs express NK-activating ligands after recognizing dsRNA [82], and cell damage has been reported to play a role in the regulation of NK-activating ligands [83]. In this manner, dsRNAs are involved in tumor damage secondary to activation of cellular effectors. Subsequently, TLR3-stimulated DCs modulate cross-priming of CD8 CTLs through incorporation of dsRNA and Ag-mounted cell debris [84]. FasL and TRAIL are major effectors for the ligands of death receptors (DRs) [85].

Soluble mediators also function as effectors in response to dsRNA. The tumor microenvironment contains many cell types and tissues, on which dsRNA and DAMP act to effect the immune response (Figure 2). Systemic administration of polyI:C induces type I IFN and enhances local T-cell immune responses in the lung and liver [48,86]. It has been postulated that polyI:C-induced type I IFN mediates the production of IL-7 [88], which promotes T-cell-derived IFN-γ to enhance macrophage recruitment and CXCR3 ligand expression [86]. NK cells are involved in early onset of IFN-γ in response to polyI:C [32] after which IFN-γ is then robustly released through IL-7 production. IL-7 is produced in the lung and liver in a type-I IFN- and IFN-γ-dependent fashion [86,87]. In addition, polyI:C-induced IL-7 promotes expression of MCP-1, contributing to recruitment of macrophages and production of CXCR3 ligands by these cells [73,88,89]. This role of polyI:C in the tumor environment defines a new mechanism by which tumor-infiltrating T/NK cells boost local T-cell immunity and by which IL-7 bridges TLR3 signal to adaptive immunity.

Our laboratory has reported that a dsRNA analog strongly activates NK cells *in vivo* [32]. Two main routes for NK cell activation have been reported. Firstly, DCs secrete several cytokines, such as IL-12, IL-18, IL-15, and IFN-α/β in response to dsRNA, and these mediators act on NK cells [33,90]. Secondly, DCs express NK-activating ligands on their cell surface which activate NK cells through cell cell contact [57]. In mouse studies, transacting IL-15 and cell-surface NK-activating ligands are crucial in polyI:C-mediated NK cell activation [33,57]. The primary NK-activating ligand induced by polyI:C is IRF-3-inducing NK-activating molecule (INAM), which contributes to NK-sensitive tumor regression [57]. In a human system with BMDC and HCV-infected debris (a source of dsRNA), NK cells are activated by BMDC via the TLR3-TICAM-1 pathway in BMDC [82]. Based on these observations, INAM may therefore participate in dsRNA-derived NK activation. It is notable that the minimal dose of dsRNA for NK activation is higher than that required for induction of type I IFN in *in vivo* systemic administration studies.

RNA-derived molecular patterns of DAMP may cause TLR3-mediated inflammation resulting from physicochemical stimuli (Figure 2). However, the functional properties of stemmed RNA generated in tumor-related inflammation have not been well demonstrated [38]. Once antigens are presented on MHC class II in DCs upon internalization of tumor cell debris, CD4 T cells [91,92], including Th1, Th2, Th9, Th17, and Tregs, are driven in a context-dependent manner. Stemmed RNA and DAMP (Table 2) may act as modifiers of this event for CD4 T cells. The induction profiling of CD4 T-cell subsets critically affects the effector-inducing capacity of myeloid DC [91], although it remains unclear whether systemic type I IFN (and the MAVS pathway) is absolutely required or not for adaptive immunity. In addition, these stimulators may serve as the second signal of TLRs triggering DCs to induce cross-presentation, which leads to mounting Ag on MHC class I and subsequently induce the proliferation of CD8 T cells (CTL) [93]. Cross-presentation is enhanced by molecules such as type I IFN and CD40, and by immune cells, including CD4 T cells, NK cells, and NKT cells [93,94]. The mechanistic role of nucleic acids sensors in the presentation of exogenous Ag by DCs remains to be determined [61]. TLR3/TICAM-1 is the main pathway for inducing cross-presentation in response to dsRNA in DCs [34]. PolyI:C or virus dsRNA is an example of a TLR3 ligand, and the cross-presentation-inducing activity of these TLR3 agonists is noticeable if sufficient amounts of polyI:C are used [8]. While the effective adjuvancy of polyI:C has been reported by Steinman *et al.* [61,91,93], no report has definitively determined the dose of RNA sufficient to promote cross-presentation and latent cross-priming (CTL-inducing) ability in humans. Further therapeutic dose analysis will provide a basis for effective strategies of dsRNA

therapy in patients who do not respond to conventional cancer therapy.

8. Expert opinion

Here, we discussed the advantages of TLR3 agonists as a therapeutic potential against cancer. TLRs generally activate transcription factors, NF-κB, which closely associates with protumor activity, thereby application of TLR agonists to adjuvant immunotherapy for cancer treatment having been controversial. TLR3 is particular in the TLR family receptors because it is not involved in MyD88 activation but only in TICAM-1 for IRF-3/7 activation, which results in production of type I and III IFNs. TLR3 is localized to the endosomal membrane in mouse CD8α$^+$ DC and human CD141$^+$ DC, suggesting that in viral infection, DCs phagocytoze noninfectious dsRNAs liberated from infected dead cells together with viral antigens. TLR3 in the DCs senses the internalized dsRNA to signal the IFN-inducing pathway. Epithelial cells and fibroblasts express TLR3 on the cell surface and directly sample dsRNA outside the cells, which may reflect the role of TLR3 in testing environment around the cells. Similar events might happen in tumor cells and DC surrounding microenvironment. Expression of TLR3 is up-regulated during malignant transformation, by eIF2 and RB, suggesting that many tumor cells can be modulated by their own TLR3 signal. The RIP1/3 pathway downstream of TICAM-1 can induce NF-κB activation, apoptosis or necroptosis that facilitates liberation of tumor antigen and its uptake by DC. Necroptosis secondary to RIP1/3 signal may be a representative outcome induced by tumor cell TLR3, although the protumor activity that induces tumor progression via the TLR3/TICAM-1 pathway is predicted to be negligible compared to the MyD88 pathway. Besides TLR3, RIG-I and MDA5 act as cytoplasmic sensors to induce systemic cytokine/IFN production leading to high serum cytokine levels. The most prominent side-effect induced by dsRNA or polyI:C (or LC) is a life-threatening cytokine shock. Indeed, the serum cytokine/IFN levels in WT mice treated with polyI:C are highly increased, but the levels are kept low in MAVS-/- mice, suggesting that polyI:C-mediated cytokinemia is largely attributable to the MAVS pathway. Although serum cytokines are high in TICAM-1-/- mice, NK cell activation and CTL proliferation are severely impaired in the absence of TICAM-1. Ultimately, we would predict that exclusive stimulation of TLR3 (i.e., TICAM-1) does not allow the serum cytokine/IFN levels in mice, whereas cellular immune effectors NK and CTL are sufficiently driven by TLR3-directed immunotherapy even in MAVS-/- mice. The strategies for specific targeting of TLR3 in dendritic cells without affecting MDA5/RIG-I should be developed for more efficient antitumor immunotherapy. If TLR3-targeted dsRNA therapy is established, tumor regresses without evoking either tumor progression or cytokinemia, two major side-effects by dsRNA-mediated inflammation then being cleared. If these TLR3 outputs are reproducible in human patients with cancer, dsRNA derivatives specifically directed against TLR3 will be an excellent therapeutic candidate for tumor immunotherapy as an adjuvant.

Clinical studies of polyI:CLC therapy for cancer was started on 1985. Since then, many clinical trials have been performed with polyI:C or LC. Most of them suggested that low doses of polyI:C did not always bring the patients good prognosis. This suggests that low-dose administration of dsRNA to patients, which appears sufficient for induction of type I IFN, is insufficient for induction for DC-driven NK activation and CTL proliferation. If administration of high doses of harmless dsRNA is feasible for adjuvant therapy, then patients with cancer benefit from therapeutic use of dsRNA. Development of less-toxic compounds specific for TLR3 would help patients with inoperable or drug-resistant tumors.

Declaration of interest

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Affiliation

Tsukasa Seya[†], Masahiro Azuma & Misako Matsumoto
[†]Author for correspondence
Hokkaido University,
Graduate School of Medicine,
Department of Microbiology and Immunology,
Kita-ku, Sapporo, 060-8638, Japan
Tel: +81 11 706 5073;
Fax: +81 11 706 7866;
E-mail: seya-tu@pop.med.hokudai.ac.jp

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REVIEW

Multi-Step Regulation of Interferon Induction by Hepatitis C Virus

Hiroyuki Oshiumi · Kenji Funami · Hussein H. Aly · Misako Matsumoto · Tsukasa Seya

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Abstract Acute hepatitis C virus (HCV) infection evokes several distinct innate immune responses in host, but the virus usually propagates by circumventing these responses. Although a replication intermediate double-stranded RNA is produced in infected cells, type I interferon (IFN) induction and immediate cell death are largely blocked in infected cells. In vitro studies suggested that type I and III IFNs are mainly produced in HCV-infected hepatocytes if the MAVS pathway is functional, and dysfunction of this pathway may lead to cellular permissiveness to HCV replication and production. Cellular immunity, including natural killer cell activation and antigen-specific CD8 T-cell proliferation, occurs following innate immune activation in response to HCV, but is often ineffective for eradication of HCV. Constitutive dsRNA stimulation differs in output from type I IFN therapy, which has been an authentic therapy for patients with HCV. Host innate immune responses to HCV RNA/proteins may be associated with progressive hepatic fibrosis and carcinogenesis once persistent HCV infection is established in opposition to the IFN system. Hence, innate RNA sensing exerts pivotal functions against HCV genome replication and host pathogenesis through modulation of the IFN system. Molecules participating in the RIG-I and Toll-like receptor 3 pathways are the main targets for HCV, disabling the anti-viral functions of these IFN-inducing molecules. We discuss the mechanisms that abolish type I and type III IFN production in HCV-infected cells, which may contribute to understanding the mechanism of virus persistence and resistance to the IFN therapy.

Keywords Hepatitis C virus · TLR3 · TICAM-1 (TRIF) · MAVS (IPS-1, Cardif, VISA) · Interferon-inducing pathway · Double-stranded RNA

Abbreviations

BMDC	Bone marrow-derived dendritic cells
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
IFN	Interferon
LD	Lipid droplet
MAM	Mitochondrial-associated endoplasmic reticulum membranes
MAVS	Mitochondrial antiviral signaling protein
Mf	Macrophages
mRNA	Messenger RNA
NK	Natural killer
NS	Non-structural
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
STING	Stimulator of IFN genes
TICAM-1	Toll-IL-1-homology domain-containing adaptor molecule-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	TNF-α receptor 1

MAVS has been identified as the adaptor for RIG-I and MDA5 by four independent groups, and then also known as IPS-1, Cardif or VISA (Kawai and Akira 2009). TICAM-1 has been identified as the adaptor for TLR3 and TLR4 by two independent groups, and thus also described as TRIF (Oshiumi et al. 2003). In accordance with the HUGO Gene Nomenclature Committee-approved nomenclature, here we refer to these adaptor molecules as MAVS and TICAM-1, respectively.

H. Oshiumi · K. Funami · H. H. Aly · M. Matsumoto · T. Seya (✉)
Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita, Sapporo 060-8638, Japan
e-mail: seya-tu@pop.med.hokudai.ac.jp

Introduction

Hepatitis C virus (HCV) mainly infects human hepatocytes, and triggers induction of cytokines and type I (IFN-α/β) and type III interferons (IFN-λ) (Fig. 1). Although cells expressing IFN receptors respond to the released IFN and amplify type I IFN production, IFN induction is not always robust in the infected cells due to the fact that HCV proteins inhibit host IFN-inducing pathways. IFN-stimulated genes (ISGs), such as IRF-7, MAP3K14, RIG-I, IRF-2, and IRF-1 are known to inhibit HCV replication (Schoggins et al. 2011). In particular, type III IFNs are more produced than IFN-α/β in HCV-infected hepatocytes via the mitochondrial antiviral signaling protein (MAVS) pathway to induce a set of ISGs (Thomas et al. 2012). Cytokines and chemokines are released from infected hepatocytes and myeloid cells in the liver. These mediators affect the formation of inflammatory environments and modify homeostasis of the host cell community, including the recruited bystander cells. Although these scenarios generally reflect the signs of patients with HCV, what occurs following initial virus entry into host cells remains obscure at the molecular level. HCV genome RNA is internalized via fusion and a portion of 3′-polyU/UC or 5′-triphosphate-short stem RNA acts directly as a ligand for RIG-I (Saito et al. 2008). The HCV genome functions as a messenger (m)RNA for HCV polyprotein production and, at the same time, HCV genome replicates in the cytoplasm (Lindenbach et al. 2007). Double-stranded (ds)RNA accumulating in infected cells is the main pattern molecule (PAMP) and, once liberated, provokes activation of innate immunity in myeloid cells. How host cells sense HCV RNA or dsRNA during infection and replicon transfection has been investigated, and has led to an understanding of the importance of the cytoplasmic RNA recognition pathways (Fig. 1), particularly, the MAVS pathway (Cheng et al. 2006; Li et al. 2005a, b). The current concept is that MAVS signals the kinases, TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), to phosphorylate IFN regulatory factors (IRF)-3 and IRF-7, resulting in the induction of type I IFN (Kawai and Akira 2009). Likewise, IRF-3 and nuclear factor (NF)-κB appear to participate in

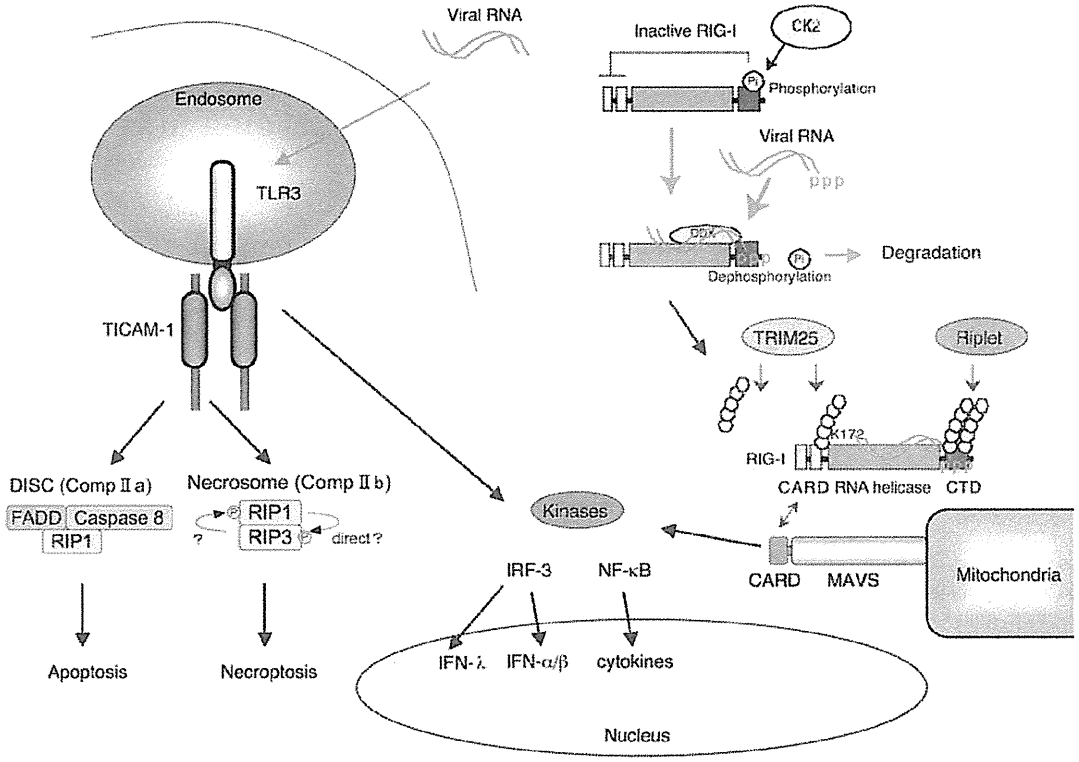

Fig. 1 Cytoplasmic and endosomal sensors for virus dsRNA in HCV infection. Live signal (*right*) and cell death signal (*left*) in response to viral dsRNA are illustrated. The live signal occurs with stimulation of TLR3 or RIG-I-like receptors and essentially induces activation of NF-κB to support induction of pro-inflammatory cytokines and type I/type III interferons (IFNs) (*right*). This live signal may be amplified by the function of a small amount of IFN-β that is required for the maintenance of homeostasis of the cellular microenvironment. In contrast, death signal occurs with TLR3: caspase 8 is a key molecule for discriminating between apoptosis and necroptosis, and its functional absence sustains the RIP1/RIP3 necrosome signal leading to necroptosis (*left*). Viral dsRNA recognition by RIG-I is induced by RIG-I ubiquitination (*right*). The two modes of K63 polyubiquitination activate RIG-I

the induction of IFN-λ (Ding et al. 2012). Because RIG-I-like receptors are IFN-inducible genes, only trace levels are found in resting cells or those in the early stage of virus entry. Thus, how RIG-I/MDA5 captures internalized or replicating virus RNA to evoke an antiviral response in such situations remains unexplained.

There are many reports suggesting that Toll-like receptor 3 (TLR3) participates in the response to HCV dsRNA (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012) (Fig. 1). Most of the relevant studies have been performed with hepatoma cell lines due to the lack of proper systems for reproducing the HCV life cycle in culture as well as the in vivo animal model to examine the HCV immune responses. Cell death accompanied with a cytopathic effect is another phenotype of infected hepatocytes (Lim et al. 2012). Hepatocyte death is characterized as apoptosis, but the possible involvement of the pathway with receptor-interacting protein (RIP) kinases in infection-induced cell death has not been strictly ruled out (Fig. 1). Necrosis-like cell death (necroptosis) might cause a source of infectious virions and lead to the pathogenesis of HCV-associated liver damage. Ligands of the death receptor family, including FasL and TRAIL, are likely to associate with hepatocyte death induced by HCV infection (Bantel and Schulze-Osthoff 2003; Saeed et al. 2011; Zhu et al. 2007); however, what triggers the induction of the effector cells is still undetermined. Apart from these cell death family proteins, it is accepted that TLR3 is an activator of the RIP1 pathway (Meylan et al. 2004), which clearly participates in macrophage necroptosis (He et al. 2011). TLR3 is up-regulated in macrophages/dendritic cells (Mf/DC) in an IFN-dependent manner (Tanabe et al. 2003) and recognizes internalized virus dsRNA in the endosome of these phagocytes (Matsumoto et al. 2011). TLR3 has been characterized as an inducer of cellular immune effectors (Matsumoto et al. 2011; Seya and Matsumoto 2009). In accordance with the current dogma, natural killer (NK)-ligand up-regulation or cross-presentation of DCs that occurs with the internalization of dead cell-derived dsRNA may bridge the missing link between HCV dsRNA and TLR3-derived DC maturation (Ebihara et al. 2008).

Dead cells are a source of damage-associated molecular pattern (DAMP) (Kono and Rock 2008). DAMP refers to an intracellular molecule with inflammation-inducing capacities when it is released out of the cell. DAMP does not belong to the cytokine family, but resembles PAMP in its functional properties toward activation of myeloid DCs and macrophages (Kono and Rock 2008). Its function may be associated with physiological responses related to HCV immune response in a broad sense, including regeneration and tumorigenesis. Recently, necrotic or necroptotic cell death has been closely connected with innate immune responses involving pattern sensing (Kono and Rock 2008; Nace et al. 2012).

Table 1 Sensors for nucleic acid PAMPs and DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5′-PPP-RNA	RIG-I
Endosomal >140 bp dsRNA	TLR3
Nonmethylated CpG DNA	TLR9
Cytosolic dsDNA	DNA sensors[a]
Self-molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4[b]
S100 proteins	RAGE
Self-nucleic acids (DAMP)	
Self-DNA	DNA sensors[a]
Self-mRNA	TLR3

HMGB1 High-mobility group box 1, *HSPs* heat shock proteins

[a] See Table 2

[b] Scavenger receptors, CD91, etc.

How HCV patterns are sensed and linked to the cellular immunity will be an intriguing issue. DAMP contains a number of cytosolic or nucleic molecules, as in Table 1, and in particular nucleic acids from infected cells. Thus, DAMP and dsRNA of viral origin are extrinsic patterns for sensors to evoke unique features of inflammation during HCV infection.

Herein, we discuss the interrelationship between these recent findings on innate immunity and HCV infection.

Blocking IFN Induction by HCV Proteins

Proteolytic Control of the IFN-Inducing Pathways by NS3/4A

HCV genome RNA serves as a single mRNA that encodes ~3,000 amino acids, consisting of 10 virus proteins (Lindenbach et al. 2007). Structural proteins (core, E1, and E2) are situated at the N-terminal region of this polyprotein (Fig. 2a). The HCV polyprotein is first cleaved between 191A and 192Y by signal peptidase to separate the core protein from E1 protein and the core is retained on the endoplasmic reticulum (ER) membrane (McLauchlan et al. 2002). Then, signal peptide peptidase scissors out the core protein by cleavage at 177F and 178L from the ER membrane (Okamoto et al. 2004). E1 and E2 are also released from the remaining structural protein complex by the proteolytic function of signal peptidase (McLauchlan et al. 2002). Non-structural proteins of HCV are fragmented into functional units by NS2 and NS3/4A proteases. Hence, the release of the structural proteins precedes the mature

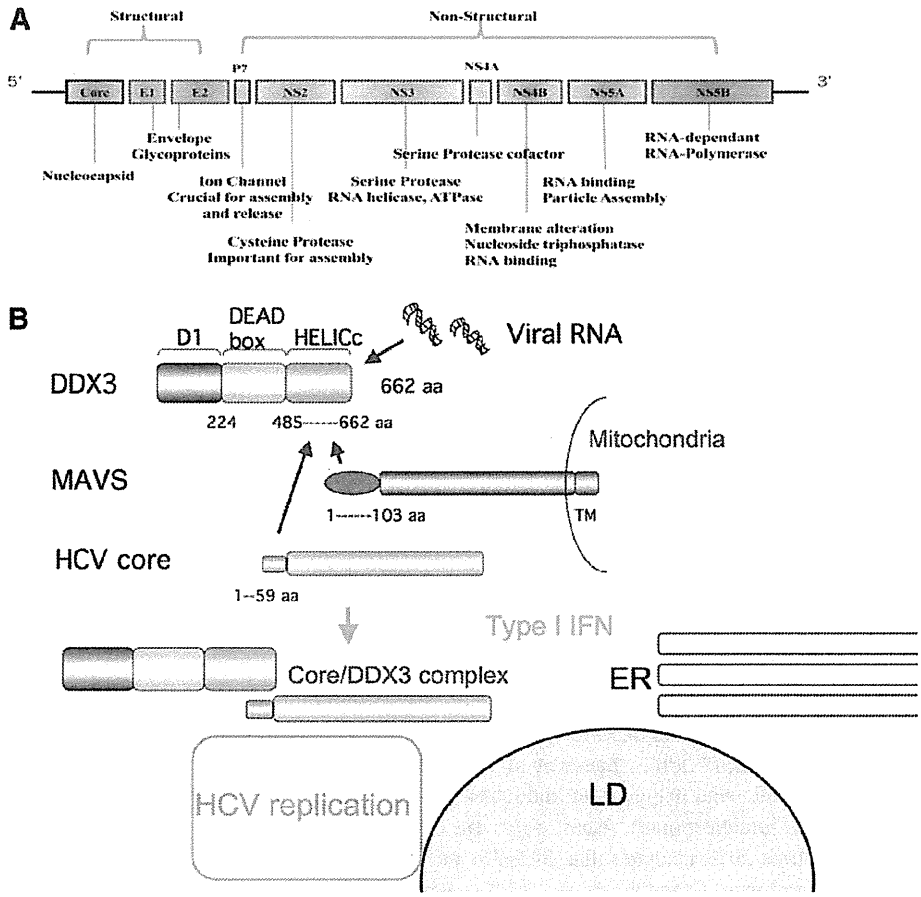

Fig. 2 Two different functions of HCV core protein. HCV genome and the functions of each HCV protein. HCV core is first clipped out from the polyprotein of HCV, and later NS proteins are generated (**a**). HCV core protein retracts DDX3 from the MAVS–DDX3 signal complex on the mitochondria (**b**). DDX3 usually couples with MAVS on mitochondria and directly binds overwhelmed virus dsRNA in virus-infected cells. When the HCV core protein is produced, DDX3 binds core protein with high affinity and moves from the mitochondria to the HCV replication apparatus, where the core is recruited. The HCV replication apparatus is situated near the lipid droplet (LD) in ER. DDX3 supports HCV replication in the apparatus

processing of non-structural (NS) proteins during the HCV polyprotein processing (Lindenbach et al. 2007). Notably, two structural proteins, core and E2, exhibit regulatory functions against type I IFN induction (Florentin et al. 2012; Mulhern and Bowie 2010).

NS3/4A protease is reported to be crucial, not only for the liberation of HCV NS proteins, but also for the regulation of host anti-viral reactions by proteolytic inactivation of host cytosolic proteins, which also interfere with homeostasis of live cells. It has been reported that NS3/4A proteolytically degrades MAVS (Cheng et al. 2006; Li et al. 2005b; Loo et al. 2006). In addition, NS4B protein has been reported to target STING to repress RIG-I-mediated type I IFN induction in hepatocytes (Nitta et al. 2012). Preceding the generation of these NS proteins, HCV core (Oshiumi et al. 2010a) and E2 proteins (Florentin et al. 2012) can suppress RIG-I-mediated type I IFN production in hepatocytes and plasmacytoid DC, respectively. In particular, the generation of core protein and NS3/4A are closely associated with suppression of the HCV-mediated host IFN biological response and the promotion of HCV replication. Since core protein is produced prior to NS3/4A in HCV-infected cells, many other functions of the core are expressed just before the proteolytic processing of HCV NS proteins within the cells.

NS3/4A cleaves MAVS (Cheng et al. 2006; Li et al. 2005b; Oshiumi et al. 2010a) and TICAM-1 (Li et al. 2005a). Hence, NS3/4A proteolytically controls at least two adaptor proteins as its substrates. In addition, Riplet is reduced in response to HCV replication (Oshiumi et al. 2010c). Whether or not Riplet is a substrate for NS3/4A is still under investigation.

MAVS Inactivation by NS3/4A

The current assumption is that the RIG-I family proteins, RIG-I and MDA5, sense viral RNA to induce type I IFN and pro-inflammatory cytokines, which in turn suppress viral infection. RIG-I and MDA5 possess the N-terminal caspase activation and recruitment domain (CARD), the central DExD/H-box helicase domain, and the C-terminal RNA-binding domain (CTD) (Fig. 1). According to crystal structure analysis, the basic region of CTD binds virus dsRNA irrespective of the presence of 5′-triphosphate (Yoneyama et al. 2004), while the CARD domain participates in interaction with the adaptor.

RIG-I recognizes HCV RNA, as well as its replication intermediate, dsRNA (Saito et al. 2008). Although MDA5 recognizes long dsRNA patterns, the role of MDA5 in HCV RNA recognition is unknown. MAVS is the key adaptor for RIG-I/MDA5-mediated IFN induction in HCV infection, although it is localized in the mitochondrial outer membrane apart from intact RIG-I molecules (Seth et al. 2005). RIG-I is not quantitatively sufficient at the protein level to capture the abundant dsRNA replicating in infected cells during the early stage; other molecules have to accept the overwhelmed dsRNA in other cytoplasmic regions (Oshiumi et al. 2010b). RIG-I is initially involved in a molecular complex (RIG-I granule), which contains many other molecules that make up a nucleocapture complex. E3 ubiquitin ligases are involved in the RIG-I granule. Ubiquitin ligases, TRIM25 (Gack et al. 2007) and Riplet (Oshiumi et al. 2009), are also situated in the RIG-I granule together with RIG-I and confer RNA-binding capacity on RIG-I through RIG-I ubiquitination (Fig. 1). TRIM25 ubiquitinates N-terminal lysines of RIG-I (Gack et al. 2007), while Riplet ubiquitinates C-terminal lysines of RIG-I (Oshiumi et al. 2009), either or both of these molecules enable RIG-I to interact with MAVS and confer mobility on mitochondria (Fig. 3). A recent report

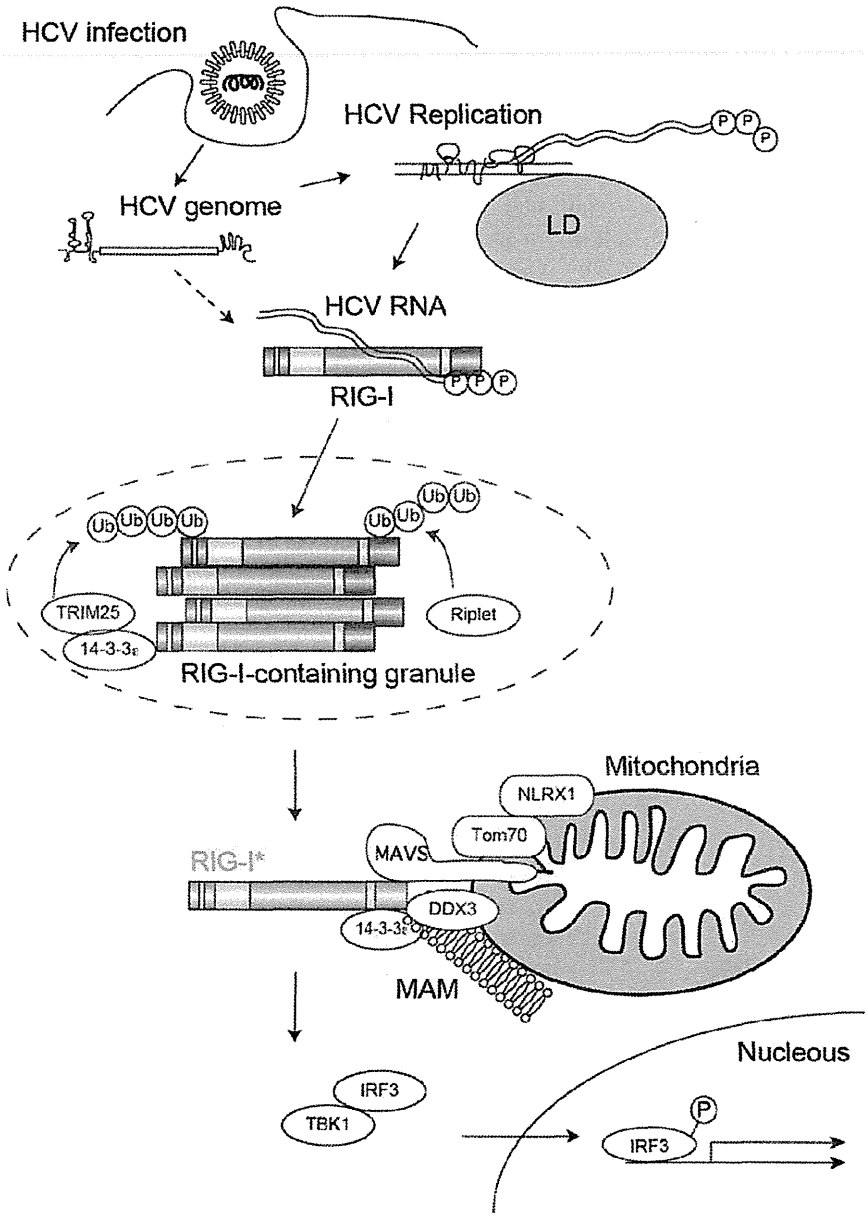

Fig. 3 Translocation of RIG-I from the cytoplasm to the mitochondria. RIG-I is diffusely distributed in the cytoplasm. When minute quantities of dsRNA enter the cytoplasm (*dashed line*), the RIG-I granule is formed with many other molecules to sense dsRNA. Once RIG-I molecules are polyubiquitinated, they form a complex with dsRNA and becomes mobile (RIG-I*). RIG-I* is recruited to the mitochondria to couple with MAVS. There are many other molecules associated with mitochondrial signaling. Because DDX3 captures overwhelmed dsRNA, the RIG-I–DDX3–MAVS complex allows robust IFN production in conjunction with dsRNA/DDX3. Whether DDX3 participates in IFN-λ induction remains undetermined

speculated that after RIG-I is up-regulated and ubiquitinated in the RIG-I granule, the 14-3-3ε is coupled with newly ubiquitinated RIG-I (Liu et al. 2012). Then, RIG-I moves from the granule to the mitochondrial membrane, a distinct membrane compartment linked to the ER, which is referred to as mitochondrial-associated endoplasmic reticulum membranes (MAM) (Horner et al. 2011). MAM accumulates MAVS and may coordinate MAVS signaling of innate immunity from peroxisomes (Dixit et al. 2010) and mitochondria (Seth et al. 2005), while MAVS localized to MAM serves as a molecular platform for the IFN-inducing signal. MAVS is constitutively complexed with DDX3, which serves as an acceptor of dsRNA in resting (RIG-I-insufficient) cells (Oshiumi et al. 2010b). If this is the case, the location for RIG-I ubiquitination (i.e., RIG-I granule) may differ from the site at which RIG-I interacts with the MAVS–DDX3 complex for signaling. Validating this issue will be of great interest in understanding initial viral RNA recognition.

Our previous data suggested that three forms of MAVS are detected in HCV-replicating hepatocyte lines by imaging analysis, as follows: intact MAVS, sequestered MAVS, and proteolytically liberated MAVS (Oshiumi et al. 2010a). These forms of MAVS simultaneously exist in hepatocytes expressing the HCV replicon or those infected with HCV. The MAVS proteolytically released from mitochondria appear to have decreased ability to activate IRF-3. MAVS is also diminished in some HCV-infected cells to lose its IFN-inducing function (Oshiumi et al. 2010a), suggesting that NS3/4A is a protease that determines the inactivation state of MAVS in HCV-replicating hepatocytes. A recent report suggested that Riplet is depleted during HCV replication (Oshiumi et al. 2010c), indicating the possibility that participation of the expressed NS3/4A protease in degrading other molecules upstream of MAVS is more important for IFN regulation than clipping out of MAVS in infected cells (Fig. 1). Similarly, other factors independent of proteolytic control may be critical for dsRNA-mediated IFN inducibility, as demonstrated by Cheng et al. (2006).

Blocking of the DDX3-Augmented IFN Production by Core Protein

Three reports have independently showed that DEAD/H Box 3 (DDX3, also known as DBX) acts as a positive regulator for MAVS-mediated type I IFN induction (Table 2; Fig. 2b). Elevation of MAVS pathway-mediated type I IFN production by DDX3 is modally different in these three reports (Mulhern and Bowie 2010). Like RIG-I and MDA5, DDX3 is a member of the DExD/H-box family of RNA helicases and is ubiquitously expressed in a variety of cells (Kim et al. 2001). The DExD/H motif of the members in this family of proteins is predictive of a role in RNA-binding and RNA-dependent cellular processing (Schroder 2009). Schroder et al. (2008) showed that the vaccinia virus protein K7 binds DDX3 and inhibits pattern-recognition receptors-induced IFN-β promoter activation. They suggested that DDX3 interacts with IKKε to enhance IRF-3 activation, while K7 counters DDX3 activity of MAVS-mediated IFN-β induction. This IFN-enhancing function of DDX3 in IRF-3 activation is located at the N-terminus of DDX3, which is the same region of the protein targeted by K7 for IRF-3 inhibition. Structure analysis of K7 complexed with a peptide from the N-terminus of DDX3 (Oda et al. 2009) has confirmed this finding.

Table 2 Nucleic acid sensors related to IFN induction in innate immunity

Pattern-recognition receptors	Adaptors	Agonists (references)	Origin
MDA5	MAVS	Cytosolic long dsRNA (Yoneyama et al. 2008)	RNA viruses
RIG-I	MAVS	Cytosolic 5′-PPP-RNA (Yoneyama et al. 2008)	RNA viruses
NOD2	MAVS	Cytosolic ssRNA (Morosky et al. 2011)	RNA viruses
TLR3	TICAM-1	Endosomal >140 bp dsRNA viruses, host (Jelinek et al. 2011)	DNA/RNA viruses
TLR7/8	MyD88	Endosomal ssRNA (Uematsu and Akira 2007)	RNA viruses, bacteria
TLR9	MyD88	Nonmethylated CpG DNA (Uematsu and Akira 2007)	RNA viruses, bacteria
DDX3	MAVS	dsRNA, ssRNA (Oshiumi et al. 2013; this review)	Viruses, host
DDX1/21, DHX36	TICAM-1	dsRNA (Rathinam and Fitzgerald 2011)	Viruses?
DDX60	MAVS	dsRNA, ssRNA, dsDNA (Oshiumi et al. 2013; this review)	Viruses, host
DHX9/DHX36 MyD88	STING	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DDX41	TBK1	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DAI (ZBP1)	STING	dsDNA (Takaoka and Taniguchi 2008)	DNA viruses
IFI16	β-Catenin	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
LRRFIP1		dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses

ssRNA single-stranded RNA

The second study indicated that DDX3 constitutively interacts with MAVS via the C-terminal region of DDX3 (Oshiumi et al. 2010b). The binding of DDX3 to MAVS is constitutive and not through the N-terminus, in contrast to the case of the virus-dependent interaction between DDX3 and IKKε (Schroder et al. 2008). RIG-I-induced IFN-β promoter reporter gene activity is inhibited by DDX3 small interfering RNA and enhanced by overexpression of DDX3 (Oshiumi et al. 2010b). Thus, DDX3 synergistically activates the IFN-β promoter together with MAVS. The C- and N-terminal regions of the DDX3 regulate MAVS-mediated IFN induction (Hogbom et al. 2007) (Fig. 1).

In contrast, Soulat et al. (2008) demonstrated a positive role for DDX3 in IFN-β promoter induction in another distinct manner. Specifically, DDX3 is shown to be a kinase substrate for TBK1 to synergistically enhance IFN-β promoter activation by TBK1. Furthermore, they demonstrated that DDX3 is recruited to the IFN-β promoter via its N-terminal region (Soulat et al. 2008). Together, these findings show that DDX3 is a positive regulator targeting the multiple sites of the RLR-induced IFN pathway. A role for DDX3 in cell cycle control and apoptosis has also been proposed in response to dsRNA (Schroder et al. 2008).

DDX3 facilitates viral replication in a variety of viruses, including HCV. The N-terminus of HCV core protein binds the C-terminus of DDX3 (Owsianka and Patel 1999). According to a recent finding, the HCV core protein participates in the suppression of DDX3-augmented MAVS signaling for IFN-β induction (Fig. 2b), which may also be related to the function of DDX3 described in the second study mentioned before (Oshiumi et al. 2010b). Unlike the DExD/H-box helicases, such as RIG-I and MDA5, DDX3 is constitutively expressed and co-localized with MAVS around mitochondria (Table 2). However, HCV core protein interferes with DDX3 function to enhance MAVS signaling by coupling with DDX3 to dissociate it from MAVS in the mitochondria. In hepatocytes with the HCV replicon, DDX3/MAVS-enhanced IFN-β-induction is largely abrogated, even when DDX3 is co-expressed. Whether DDX3 enhances IFN-λ induction like RIG-I remain untested. DDX3 is spotted with minimal merging with MAVS in confocal analysis, and partly assembles in the HCV core protein located near the lipid droplet (LD) in replicon-positive hepatocytes, although in some cells MAVS is diminished or disseminated apart from mitochondria. Thus, HCV core retracts DDX3 from MAM, where RIG-I moves from the RIG-I granule to assemble together with MAVS (Figs. 2b, 3).

Our consensus is that the binding of HCV core protein to DDX3 and suppressing DDX3–MAVS complex formation are crucial for inhibition of the MAVS pathway. However, multiple functions of DDX3 may be reflected in other functional aspects of core protein; specifically, the DDX3–core interaction is required for HCV replication (Ariumi et al. 2007). Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes appear independent of its interaction with the viral core protein (Angus et al. 2010). In addition, the association between DDX3 and core protein implicates DDX3 in HCV-related hepatocellular carcinoma progression (Chang et al. 2006). Based on its core protein association and MAVS-regulatory properties, DDX3 appears to be switched by the core protein from an HCV-suppressing (i.e., IFN-inducing) mode to an HCV replication-supporting mode (Fig. 2b). The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

Evidence is accumulating that HCV assesses many steps in the IFN-inducing pathway throughout the early and late infection stages, and suppresses IFN production by multiple means. Disruption of MAVS function by NS3/4A and core protein may be crucial in HCV-infected Huh7.5 cells, even though the cells harbor dysfunctional RIG-I (Binder et al. 2007). Type I IFN suppresses tumor progression by causing expression of p53 and other tumor-suppressing agents (Takaoka et al. 2005). E2 and NS4B may affect tumor progression by controlling type III IFN induction. These unique features of the HCV proteins require further confirmation and should be in the focus of investigation regarding HCV persistence, chronic infection, and progression to cirrhosis and carcinoma.

Inactivation of the TICAM-1 Pathway by NS3/4A

TICAM-1 pathway has been associated with chemokine production, apoptosis, necroptosis, and IL-12p40 production in hepatoma cell lines expressing TLR3 (Li et al. 2012); however, such immune responses are predominantly absent in primary cultured cells. This might be explained by the TLR3 signaling that is likely to be shut off in most normal hepatocytes, but executed in hepatocytes of the infectious liver during chronic states of HCV infection or exposure to dsRNA stimulation. Despite the constitutive expression of the adaptor molecule TICAM-1 in human hepatocytes, only trace amount of TLR3 is being expressed in comparison to RIG-I that is commonly expressed. This gives us an insight of the role played by other cytoplasmic dsRNA sensors such as DDX1/DDX21/DHX36 (Table 2), in the activation of TICAM-1 pathway (Zhang et al. 2011). It remains unknown whether these cytoplasmic dsRNA sensors participate in IFN-λ induction.

Although there are positive findings supporting the importance of TLR3 in the pathogenesis of HCV infection (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012), the expression level of TLR3 is still a contentious issue. TLR3 protein is undetectable by immunostaining with monoclonal antibody (TLR3.7) against huTLR3 in

uninfected human hepatocytes (Nakamura et al. 2008) or Huh-7 hepatoma cells that are commonly used for propagation of HCV (Wang et al. 2009). Only a few reports have shown evidence that TLR3 protein is weakly detected in resting primary cultured hepatocytes (Wang et al. 2009). On the other hand, several reports have suggested that the TLR3 at the messenger level was observed in cultured hepatocytes and hepatoma cell lines by real-time polymerase chain reaction (Khvalevsky et al. 2007). Since TLR3 expression is partly regulated by p53, mutated p53 in Huh-7 cells as well as other hepatocellular carcinoma cell lines may attribute to the specific lacking of the TLR3 expression. In addition, Tanabe et al. (2003) demonstrated that the lack of TLR3 expression in Huh-7 cells may be due to other transcriptional regulations.

TLR3 expression appears to be up-regulated in cultured hepatocytes in response to polyI:C (Wang et al. 2009). Similarly, TLR3 is up-regulated in hepatocytes of patients with chronic HCV infection or polyI:C-injected mice (McCartney et al. 2009; Nakamura et al. 2008). These results lead to the assumption that TLR3 can be up-regulated in hepatocytes in an infectious milieu in response to the produced dsRNA or liberated type I IFN by HCV-infected hepatocytes via the RIG-I-mediated IFN-inducing pathway. A similar mechanism of secondary induction of the TLR3 expression by type I IFN may as well occur in hepatoma cells. Thus, HCV infection or malignant transformation allows hepatocytes to turn TLR3 positive, enabling the activation of the TICAM-1 pathway by external virus dsRNA. This would explain the finding that TLR3 is highly expressed in biliary epithelial cells and certain hepatoma cell lines (Harada et al. 2007; Nakamura et al. 2008). Li et al. (2012) showed that TLR3 senses HCV infection and induces ISG expression when TLR3 is over-expressed artificially in the Huh7.5 cells that are deficient in RIG-I signaling. They demonstrated that HCV replication is partially restricted when the cells are infected at a low multiplicity. However, such protective effect is dismissive when the infection is overwhelmed at high multiplicity partly due to the limitation of the TLR3.

TLR3 has a distinct feature from RIG-I as TLR3 can potentially sense viral dsRNA released into the extracellular environment by other cells. Considering the fact that NS3/4A protease that interferes with the host anti-viral reactions is only expressed within infected cells, TLR3-mediated immune responses might be triggered in the uninfected hepatocytes or other cell types. On top of that, it has been reported that TICAM-1 is also a substrate for NS3/4A protease in hepatoma cell lines (Li et al. 2005a). Ferreon et al. (2005), has confirmed this in the corollary biochemical studies. TICAM-1 and its signal pathway are intact within the cells around infected hepatocytes (Shimoda et al. 2011). This may contribute to the IFN responses observed in some patients.

TLR3 is highly expressed in biliary epithelial cells where biliary atresia occurs in response to the interaction between TLR3-stimulated monocytes and liver NK cells (Harada et al. 2007; Shimoda et al. 2008, 2011). However, it remains intriguing whether the up-regulated TRAIL or FasL in NK cells resulted from the IFN-signaling (Estornes et al. 2012) are responsible for the induction of cell death in hepatocytes. Nevertheless, TICAM-1-mediated cell death in some HCV-infected hepatocytes is also likely to occur via autophagy or *trans*-acting of dsRNA generated by HCV replication.

DAMP and dsRNA in HCV Pathology

Live or death signals are usually raised by viral dsRNA in virus-infected cells. Type I/type III IFNs and proinflammatory cytokines (IL-6, IL-12, TNF-α etc.) are liberated through IRF-3/7 and NF-κB activation as the output from virus-infected cells that are alive. In contrast, DAMP and cytoplasmic cytokines converted to active forms by caspase 1 eventually result from activation of inflammasome and often links to cell death events (Yeretssian 2012). TLR, Nod-like receptor and other cytosolic nucleic acid sensors are closely associated with PAMP/DAMP recognition (Table 2), therefore inflammation states are fundamentally modified by these factors (Bortoluci and Medzhitov 2010).

Replication of virus RNA allows hepatocytes to induce type III IFN, IL-7 and chemokines (Apolinario et al. 2005; Zeremski et al. 2007). HCV also regulates production of chemokines (Sillanpaa et al. 2008) and type III IFNs (Thomas et al. 2012) by infected cells. Polymorphisms around the IL-28B gene have been associated with clearance of HCV in human, indicating a role for type III IFNs rather than type I in HCV infection (Thomas et al. 2012), although little is known about the function of type III IFNs in intrinsic antiviral responses. IFNs and IL-7 released from HCV-infected hepatocytes possibly act on myeloid cells and lymphocytes expressing their receptors to induce IFN-γ (Sawa et al. 2009). Once type I/type III IFN and IFN-γ are systemically distributed, synergistic function of these IFNs allows the systemic cells to produce ISGs including CXCL10 (IP-10) and CCL5 (RANTES) (Larrubia et al. 2008; Zeremski et al. 2007). In addition, dsRNA-induced IL-7 forms a positive amplifying loop with T-cell-derived IFN-γ to promote macrophage recruitment and CXCR3 ligand (CXCL10) expression by these macrophages (Andersson et al. 2009). Since CXCR3 is mainly expressed on activated T and NK cells, these cytotoxic effectors gather around the inflammatory nest as well as secondary affected organs. HCV-related extrahepatic

disorders are likely to occur in conjunction with ectopic T-cell immune response (Antonelli et al. 2008, 2009). In addition, these immunological aberrances may be modulated by viral factors. In fact, in mouse models, NS5A expression impairs clearance of other viruses from the liver due to the inhibition of IFN-γ production (Kanda et al. 2009). By any means, induction of IFN-γ in concert with activation of cellular immunity is a major array for live signal in HCV infection.

Necrosis-like cell death occurs contrarily in a cell type-specific manner as a result of death signal. Tumor necrosis factor (TNF)-α and its receptor, TNFR1, are implicated in this process. The coupling of RIP1 with RIP3, termed a necrosome, is responsible for the switching of apoptosis to necroptosis (Cho et al. 2009; He et al. 2009). Caspase 8 acts as a key protease for blocking the formation of the necrosome; the RIP1/RIP3 complex can assemble only in the absence of functional caspase 8. It has been reported that virus dsRNA often induces apoptosis in infected cells, which is known as cytopathic effect (Lim et al. 2012). TICAM-1 and RIP1 may be involved in the virus-derived apoptosis. Yet, possible involvement of RIG-I/MDA5 in cell death cannot be ruled out in some cases of viral infection (Eksioglu et al. 2011). In HCV-infected hepatocytes, how the TLR3/TICAM-1 pathway is involved in necroptotic inflammation is the next issue to be elucidated with respect to HCV pathogenesis (Fig. 1). HCV dsRNA and DAMP can be liberated from infectious hepatocytes, as well as virus particles. TNF-α and IL-6 are the pro-inflammatory cytokines released during HCV infection. Hence, a characteristic feature of the HCV-infected hepatocytes is that DAMP is released together with viral dsRNA from necroptotic HCV-infected cells to the surrounded environment. These factors stimulate nucleic acid sensors of myeloid DC/Mf in the draining region (Table 2). We expect that necroptosis will be of enormous interest in HCV infection following smoldering inflammation. Because each virus species harbors distinct strategies for escaping the innate dsRNA-sensing system, the physiological role of TLR3-mediated necroptosis should be analyzed in a HCV-specific fashion.

Cellular Immunity Induced by HCV-Infected Cell Debris

Once DC/Mf responds to these unusual innate stimulators, DAMP and dsRNA, cellular immunity is provoked against HCV Ag with irregular modification by these immune enhancers during HCV infection. NK cells and cytotoxic T lymphocytes (CTL) are known to be driven in myeloid DCs by stimulation with dsRNA, and in fact are the main effectors against HCV-infected hepatocytes based on several different systems (Ebihara et al. 2008; Saeed et al. 2011; Zhu et al. 2007). DCs express NK-activating ligands by recognizing dsRNA to activate NK cells (Ebihara et al. 2007), and cell damage is reported to play a role in the regulation of NK-activating ligands (Wen et al. 2008), thereby dsRNA and DAMP are involved in the elimination of HCV-infected cells. Subsequently, DCs cross-prime CD8 CTLs through incorporation of dsRNA and HCV Ag-mounted cell debris (Jin et al. 2007). FasL and TRAIL are major effectors for the ligands of death receptors. HCV-infected cells will be eliminated if the cells express high levels of MHC class I with HCV antigen.

Our laboratory has reported that a dsRNA analog (polyI:C) has strong ability to activate NK cells in vivo (Akazawa et al. 2007; Matsumoto and Seya 2008). Two main routes for NK cell activation have been reported. First, DCs secrete several cytokines, such as IL-12, IL-18, IL-15 and IFN-α/β in response to dsRNA, and these mediators act on NK cells (Lucas et al. 2007; Matsumoto et al. 2011). Second, DCs express NK-activating ligands on their cell surface and the ligands make a balance shift to activation of NK cells through cell–cell contact (Ebihara et al. 2010). In mouse studies, IL-15 and cell-surface NK-activating ligands are crucial in polyI:C-mediated NK cell activation (Ebihara et al. 2010; Lucas et al. 2007). A main NK-activating ligand induced by polyI:C is IRF-3-inducing NK-activating molecule (INAM) (Ebihara et al. 2010). In a human system with bone marrow-derived DCs (BMDC) and HCV-infected debris (a source of dsRNA), NK cells are activated by BMDC via the TLR3-TICAM-1 pathway in BMDC (Ebihara et al. 2008). Thus, INAM may be a factor that participates in HCV-derived NK activation.

However, how dsRNA and DAMP modify the maturation of DC in an infectious milieu to induce CD4 and CD8 T cells is largely unknown because the functional properties of DAMP generated in HCV-infected hepatocytes have not been well documented (Azuma et al. 2012). Once antigens are presented on MHC class II in DCs upon internalization of infectious debris, CD4 T cells (Longhi et al. 2009), including Th1, Th2, Th9, Th17, and Tregs, are driven in a sophisticated manner. In this context, DAMP and dsRNA could act as the second signal of TLRs triggering DCs to induce cross-presentation, which leads to mounting Ag on MHC class I and subsequently induce the proliferation of CD8 T cells (CTL) (Caskey et al. 2011). Furthermore, the so-called innate lymphocytes may respond to intrinsic stimuli in an Ag-independent fashion. Thus, the function of nucleic acid sensors for DAMP and dsRNA in the presentation of exogenous antigen by DCs is an issue to be tackled (Caskey et al. 2011). Cross-presentation is enhanced by molecules, such as type I IFN and CD40, and by immune cells, including CD4 T cells, NK cells, and NKT cells (Matsumoto and Seya 2008). Yet, the role of type III IFN in T-cell cross-priming and innate lymphocyte activation are

yet unknown. TLR3/TICAM-1 is a main pathway for inducing cross-presentation in response to dsRNA in DCs (Azuma et al. 2012). PolyI:C or virus dsRNA is an example of the TLR3 ligand, and their cross-presentation-inducing activity was first described by Schulz et al. (2005). The effective adjuvancy of polyI:C has been subsequently reported by Steinman group (Caskey et al. 2011; Longhi et al. 2009); however, no report has been definitively determined which DAMPs participate in cross-presentation and possess latent cross-priming (CTL-inducing) ability.

Why HCV circumvents the host immune system of both innate and acquired arms of the immune system remains an ambiguous question. Hepatocytes stand with unique properties with lipid droplet (LD) and the bile secretion system, where hepatocytes secrete bile into canaliculi, which flows into choledochus. HCV and hepatitis B virus induce smoldering inflammation, which is believed to be a nest of carcinogenesis. These viruses have no common properties in their viral-side factors, but the host factors including hepatocytes are common bases for triggering inflammation. Thus, the host factors are undoubtedly critical in inducing infection-driven inflammation and perhaps initiation of tumor progression (Seya et al. 2012). We speculate that persistent HCV infection, followed by inflammation, is caused by the immune aberration involved in HCV infections. The main factors in the innate arm of immunity are DAMP and dsRNA, each of which is reported to associate with smoldering inflammation. However, it is unknown what occurs in the liver if this combined stimulation is constitutively exerted in HCV-infected cells and myeloid cells in the liver. Examining the function of dsRNA and DAMP on chronic HCV infection with increasing studies in innate immunity, inflammation, and cell death, will help us extending our knowledge on vaccine and adjuvants against HCV infection and tumorigenesis. Further molecular analysis will provide a hint for therapeutic strategies for patients who do not respond to IFN therapy.

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